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INTRODUCTION

BACKGROUND

The role of erbB receptors in breast cancer. The establishment and progression of breast cancer is controlled by receptors for peptide growth factors and for estrogens (1). Of these receptors, the Epidermal Growth Factor Receptor (EGFR) family has been heavily implicated in breast cancer. This type I receptor tyrosine kinase (RTK) family consists of four receptors: EGFR (erbB1), Human Epidermal growth factor Receptor 2 (HER-2, erbB2), HER-3 (erbB3), and HER-4 (erbB4). These RTKs are transmembrane cell surface glycoproteins that either homo- or hetero-dimerize to signal. The signal transduction cascade commences with growth factor binding, receptor dimerization, and tyrosine autophosphorylation of the cytoplasmic domain. This leads to the recruitment of intracellular signaling effectors that activate the MAPK mitogenic signaling cascade and the AKT survival-signaling cascade(2-4).

The erbB growth factor receptors are under tight control in the cell. Disruption of this control leads to aberrant signaling and growth, and converts estrogen-responsive cells to an estrogen-independent state(5). Overexpression of HER-2 is a common molecular abnormality in breast cancer, implicated in 20 - 30% of all cases(6). HER-2 is the preferred heterodimer partner of the erbB receptors. This heterodimerization capacity leads to increased signaling diversity and oncogenic capability, resulting in a pathogenic role for these receptors in breast cancer(7-10).

ErbB directed breast cancer therapies. Due to the erbB receptors' prominent role in carcinogenesis, they have become prime targets for cancer therapies. There are currently several studies on small molecule inhibitors that specifically target the cytoplasmic domain of the EGFR, including ZD1839 and AG1478(11-14). Herceptin, an inhibitory antibody, specifically targets the ectodomain of HER-2(15). However, its efficacy is limited to a subset of HER-2 overexpressing breast tumors, and its molecular mechanism is poorly understood. This points to the great need of additional erbB-directed therapies such as Herstatin.

Herstatin, a novel erbB receptor inhibitor. Herstatin was recently discovered in the Laboratory of Dr. Gail M. Clinton as an alternate transcript of the HER-2 gene(16). This transcript is created by intron retention and encodes a truncated HER-2 protein. Herstatin consists of subdomains I and II of the HER-2 receptor ectodomain, and a novel intron-encoded c-terminal domain. Expression of herstatin is extremely low in breast carcinoma cells with elevated HER-2 expression(16). Herstatin binds with high affinity to the ectodomains of HER-2 and EGFR and blocks dimerization and tyrosine phosphorylation of these receptors(16-18). The ability of herstatin to disrupt the obligate first steps in receptor activation from outside the cell points to its potential as a cancer therapeutic.

PRELIMINARY STUDIES

The intron-encoded domain of herstatin is a novel binding module. Preliminary efforts to examine the individual subdomains of herstatin have yielded evidence of the direct interaction of the intron-encoded domain (int8hst) with the ectodomains of EGFR and HER-2. We have shown that int8hst binds specifically to HER-2 overexpressing, but not to parental NIH-3T3 cells (Figure 1A). Additionally, we have shown that int8hst binds to EGFR-3T3 cells in the presence and absence of EGF, thus suggesting that binding of int8hst is independent of the activation state of the receptor (Figure 1B).

Figure 1.

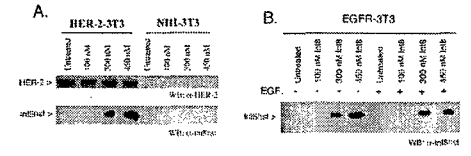

Figure 1. A. Recombinant int8hst binds specifically to HER-2 overexpressing, but not parental 3T3 cells. B. Recominant int8hst binds to EGFR overexpressing 3T3 cells in the presence and absence of EGF.

Recent studies have shown the crystal structures of the ectodomains of many erbB receptor family members(15,19-22). These studies have revealed a dimerization scheme for EGFR by which the dimmers form in a back-to-back fashion, with extracellular inter-receptor contacts being mediated by subdomains II and IV(20,22). Of importance is a conserved loop in subdomain II that serves as an energetically weak dimerization arm, stabilized by other contacts within the receptor. It is interesting to note that subdomain II is present in herstatin, indicating

that herstatin also contains the conserved dimerization loop. Binding of herstatin may then be conferred by the intron-encoded domain and stabilized by the dimerization of arm present in subdomain II.

The intron-encoded domain confers HER-2 specific binding to heterologous protein complexes. In order to further examine the role of the intron-encoded domain as a binding module, we asked whether int8hst could confer HER-2 specific binding to heterologous protein complexes such as *Cow Pea Mosiac Virus* (CPMV). The N-terminus of int8hst was chemically crosslinked through a thioester linkage to the surface of the CPMV capsid (CPMV-int8hst). This was done in collaboration with Dr. Jack Johnson, Dept. of Molecular Biology, The Scripps Research Institute, La Jolla, CA. Previous analysis has shown that this crosslinking occurs on an exposed lysine, K234, of the large subunit on the surface of CPMV. CPMV-int8hst bound specifically to HER-2 overexpressing, but not to parental NIH-3T3 cells. Additionally, this binding was not seen with wild-type CPMV, suggesting that the intron-encoded domain, int8hst, conferred HER-2 specific binding to CPMV.

Figure 2.

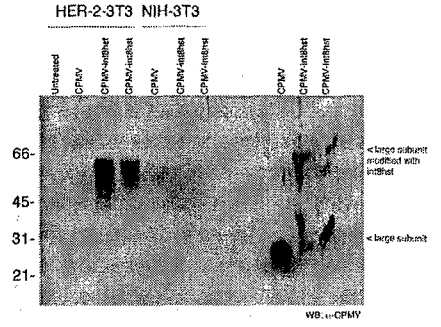

Figure 2. The intron-encoded domain (int8hst) confers HER-2 specific binding to CPMV. CPMV-int8hst binds specifically to HER-2 overexpressing, but not parental NIH-3T3 cells.

Full-length herstatin has bioactivity. Recombinant herstatin has been produced and purified from Drosophila S2 cells. Dose response studies of insect herstatin against human carcinoma cells indicate an IC50 of about 10-20nM. The growth-inhibitory activity of purified herstatin has been demonstrated in HER-2 dependant breast carcinoma (SKBR3, BT474, and MCF7/HER-2) cell lines(23).

BODY

Retention of intron 8 in alternative HER-2 mRNA generates an inhibitory secreted ligand, herstatin, with a novel receptor-binding domain (RBD) encoded by the intron. This study examines binding interactions with several receptors and investigates sequence variations in intron 8. The RBD, expressed as a peptide, binds at nM concentrations to HER-2, the EGFR, ΔEGFR, HER-4 and to the IGF-1 receptor, but not to HER-3 nor to the FGF-3 receptor, whereas a rare mutation in the RBD (Arg to Ile) eliminates receptor binding. The full length herstatin binds with 3-4 fold higher affinity than its RBD, but with ~10 fold lower affinity to the IGF-IR. Sequence conservation in rhesus monkey but not in rat suggests that intron 8 recently diverged as a receptor-binding module critical for the function of herstatin. (See Shamieh, et al. *FEBS Letts.* 568 (2004) in appendix.)

Previous studies have shown that herstatin binds to the ectodomain of multiple members of the EGF receptor (EGFR) family, and that binding to EGFR and HER-2 ($K_d \approx 15$ nM) blocks receptor dimerization and ligand activation. Furthermore, herstatin was recently found to also bind to the IGF-I receptor (IGF-IR) ($K_d \approx 150$ nM), which exhibits signaling crosstalk and contains regions of high homology with the ectodomain of the EGFR family (24). We, therefore, investigated the impact of herstatin expression on IGF-I signaling and proliferation in parental and herstatin-transfected MCF7 breast cancer cells. IGF-IR levels, as well as IGF-I-mediated IGF-IR tyrosine phosphorylation, were reduced several-fold in two different clonal isolates of herstatin-expressing cells. Down-regulation did not appear to be caused by herstatin-mediated inhibition of the EGFR, since treatment of parental MCF7 cells with an EGFR-specific inhibitor, AG1478, for up to 24 hours did not reduce IGF-IR levels. Examination of the impact of herstatin on IGF-I-specific signaling revealed strong inhibition of tyrosine phosphorylation of IRS-1, while IRS-2 activation was enhanced. Although IGF-IR tyrosine phosphorylation was strongly reduced, herstatin expression did not inhibit, but stimulated, IGF-I-mediated ERK activation, while IGF-I activation of the PI3K-Akt/PKB pathway was inhibited. Altered IGF-IR signaling culminated in loss of IGF-I-mediated cell growth and survival in herstatin-expressing clonal cell lines. These studies demonstrate that herstatin profoundly modulates IGF-I-stimulated signaling and proliferation in MCF7 breast cancer cells, either through direct interaction with the IGF-IR or indirectly by modulating crosstalk with the EGFR family. (See Shamieh et al. *manuscript submitted to JBD* in appendix.)

KEY RESEARCH ACCOMPLISHEMENTS AND REPORTABLE OUTCOMES

- **Shamieh, L.S.,** Carroll, J.M., Hart, E, Clinton, G.M., and Roberts, C.T. Modulation of insulin-like growth factor signaling by herstatin, an alternatively spliced HER-2 (erbB-2) gene product. *(Manuscript submitted – Journal of Biological Chemistry)*

- **Shamieh, L.S.,** Evans, A.J., Denton, M.C., and Clinton, G.M. Receptor binding specificities of herstatin and its intron 8-encoded domain. ***FEBS Lett.*** 568(1-3): 163-6 *(2004)*

CONCLUSIONS

Herstatin binds to both EGF and IGF-I receptor families and may function as a multi-functional inhibitor

It has been shown that herstatin binds to EGFR and HER-2 and inhibits dimerization and receptor activation (16,18). Furthermore, studies have also shown that herstatin expression inhibits Hrg and EGFmediated signaling (17,18,25). These studies suggest that the binding of herstatin, which is a secreted, soluble piece of the HER-2 ectodomain, is integral to its inhibitory function. However, previous studies have shown that a membrane-anchor is required for dimerization between ectodomains or pieces of ectodomains of erbB receptors (26). This dependence on a membrane-anchor suggests that the energetics of soluble receptor ectodomains alone are not strong enough to mediate dimerization (21,26). An increase in effective local concentration is not enough to overcome this requirement of a membrane-anchor, as there is a marked absence of HER-2 ectodomain dimers in the crystal structure (21). Since herstatin is not membrane-bound but still binds to EGFR and HER-2 with nanomolar affinity, I propose that the intron 8-encoded domain may facilitate binding of herstatin to the receptor ectodomain, overcoming the requirement for membrane immobilization.

Because of the importance of binding function of herstatin for its inhibitory activity and because of the novel sequence of the intron encoded binding module, I considered it important to define the diversity of receptors to which herstatin binds and to determine the strength of the binding interactions. I investigated whether herstatin binds to other members of the EGF receptor family. In addition to binding to EGFR and HER-2, I found that herstatin also binds to HER-3, HER-4, that is to all members of the EGF receptor family in contrast to any of the other 11 erbB ligands. This suggests that these receptors are all targets of herstatins' inhibitory effects and that there may be a common herstatin binding site in the ectdomain of all erbB receptors. I propose that heteromeric interactions between subdomains I and II of the receptor ECDs and herstatin function to stabilize the binding of herstatin, thus resulting in a lower K_d than binding of int8 alone.

In addition to binding to the EGF receptor family, I found that herstatin binds, albeit with reduced affinity, (10 fold) to the IGF-IR. The stoichiometry of herstatin binding to the IGF-I receptor family was one herstatin molecule to one receptor dimer, as opposed to the stoichiometry of binding to the EGF receptor family (one herstatin molecule to one receptor monomer). This difference in stoiciometry may be due to steric clash between subdomains I and II of herstatin and the disulphide-bonded IGF-I receptor family ECD, and may reflect differences in herstatin's ability to regulate the two receptor families. This thesis provides the first evidence that herstatin binds to two, independent receptor tyrosine kinase families: the EGFR family and the IGF-IR family.

Herstatin not only binds to the IGF-IR family, but also inhibits IGF-I-induced signaling and growth in breast carcinoma cells. Previous studies have shown that herstatin also inhibits EGF and Hrg-induced signaling and growth in a variety of cells (17,18,25). Due to its involvement in carcinogenesis, the EGF receptor family has been a target of anti-cancer directed therapies. Recent evidence has shown that the inhibitory effects of Iressa, an EGFR small molecule inhibitor, and Herceptin, an anti-HER-2 monoclonal antibody, can be overcome by IGF-I signaling through the EGF receptor (27-29). I suggest that herstatin, which binds to both the EGF and IGF-I receptor families, may have potential as a multi-functional therapeutic.

REFERENCES

1. Nass, S. J., and Davidson, N. E. (1999) *Hematol Oncol Clin North Am* **13**(2), 311-332
2. Dougall, W. C., Qian, X., Peterson, N. C., Miller, M. J., Samanta, A., and Greene, M. I. (1994) *Oncogene* **9**(8), 2109-2123
3. Hynes, N. E., and Stern, D. F. (1994) *Biochim Biophys Acta* **1198**(2-3), 165-184
4. Klapper, L. N., Glathe, S., Vaisman, N., Hynes, N. E., Andrews, G. C., Sela, M., and Yarden, Y. (1999) *Proc Natl Acad Sci U S A* **96**(9), 4995-5000
5. Dowsett, M. (2001) *Endocr Relat Cancer* **8**(3), 191-195
6. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and et al. (1989) *Science* **244**(4905), 707-712
7. Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) *Nature* **366**(6454), 473-475
8. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., 3rd. (1994) *J Biol Chem* **269**(20), 14661-14665
9. Karunagaran, D., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. (1995) *J Biol Chem* **270**(17), 9982-9990
10. Karunagaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E., and Yarden, Y. (1996) *Embo J* **15**(2), 254-264
11. Wakeling, A. E., Barker, A. J., Davies, D. H., Brown, D. S., Green, L. R., Cartlidge, S. A., and Woodburn, J. R. (1996) *Breast Cancer Res Treat* **38**(1), 67-73
12. Arteaga, C. L., Ramsey, T. T., Shawver, L. K., and Guyer, C. A. (1997) *J Biol Chem* **272**(37), 23247-23254
13. Barker, A. J., Gibson, K. H., Grundy, W., Godfrey, A. A., Barlow, J. J., Healy, M. P., Woodburn, J. R., Ashton, S. E., Curry, B. J., Scarlett, L., Henthorn, L., and Richards, L. (2001) *Bioorg Med Chem Lett* **11**(14), 1911-1914
14. Kurokawa, H., and Arteaga, C. L. (2001) *Clin Cancer Res* **7**(12 Suppl), 4436s-4442s; discussion 4411s-4412s
15. Cho, H. S., Mason, K., Ramyar, K. X., Stanley, A. M., Gabelli, S. B., Denney, D. W., Jr., and Leahy, D. J. (2003) *Nature* **421**(6924), 756-760
16. Doherty, J. K., Bond, C., Jardim, A., Adelman, J. P., and Clinton, G. M. (1999) *Proc Natl Acad Sci U S A* **96**(19), 10869-10874
17. Justman, Q. A., and Clinton, G. M. (2002) *J Biol Chem* **277**(23), 20618-20624
18. Azios, N. G., Romero, F. J., Denton, M. C., Doherty, J. K., and Clinton, G. M. (2001) *Oncogene* **20**(37), 5199-5209
19. Cho, H. S., and Leahy, D. J. (2002) *Science* **297**(5585), 1330-1333
20. Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Zhu, H. J., Walker, F., Frenkel, M. J., Hoyne, P. A., Jorissen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (2002) *Cell* **110**(6), 763-773
21. Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Kofler, M., Jorissen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (2003) *Mol Cell* **11**(2), 495-505
22. Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J. H., Saito, K., Sakamoto, A., Inoue, M., Shirouzu, M., and Yokoyama, S. (2002) *Cell* **110**(6), 775-787

23. Guo, S. A. J. E., M.C. Denton, L. Shamieh, J. Rhodes, and G.M. Clinton. (2002) *Proc Am Assoc Cancer Res* **43**, 1004
24. Adams, T. E., McKern, N. M., and Ward, C. W. (2004) *Growth Factors* **22**(2), 89-95
25. Jhabvala-Romero, F., Evans, A., Guo, S., Denton, M., and Clinton, G. M. (2003) *Oncogene* **22**(50), 8178-8186
26. Kumagai, T., Davis, J. G., Horie, T., O'Rourke, D. M., and Greene, M. I. (2001) *Proc Natl Acad Sci U S A* **98**(10), 5526-5531
27. Camp, E. R., Summy, J., Bauer, T. W., Liu, W., Gallick, G. E., and Ellis, L. M. (2005) *Clin Cancer Res* **11**(1), 397-405
28. Jones, H. E., Goddard, L., Gee, J. M., Hiscox, S., Rubini, M., Barrow, D., Knowlden, J. M., Williams, S., Wakeling, A. E., and Nicholson, R. I. (2004) *Endocr Relat Cancer* **11**(4), 793-814
29. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D., and Pollak, M. (2001) *J Natl Cancer Inst* **93**(24), 1852-1857

MODULATION OF INSULIN-LIKE GROWTH FACTOR SIGNALING BY HERSTATIN, AN ALTERNATIVELY SPLICED HER-2 (erbB-2) GENE PRODUCT

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Running Title: Herstatin regulation of IGF signaling

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Herstatin, a product of alternative splicing of the HER-2 gene, consists of subdomains I and II of the ectodomain of the HER-2 receptor tyrosine kinase, followed by a 79-amino acid C-terminal domain encoded by intron 8. Previous studies have shown that herstatin binds to the ectodomain of multiple members of the EGF receptor (EGFR) family, and that binding to EGFR and HER-2 blocks receptor dimerization and ligand activation. Herstatin was recently found to also bind to the IGF-I receptor (IGF-IR), which exhibits signaling crosstalk and contains regions of high homology with the ectodomain of the EGFR family (1). We, therefore, investigated the impact of herstatin expression on IGF-I signaling and proliferation in parental and herstatin-transfected MCF7 breast cancer cells. IGF-IR levels, as well as IGF-I-mediated IGF-IR tyrosine phosphorylation, were reduced several-fold in two different clones of herstatin-expressing cells. Down-regulation did not appear to be caused by herstatin-mediated inhibition of the EGFR, since treatment of parental MCF7 cells with an EGFR-specific inhibitor, AG1478, for up to 24 hours did not affect IGF-IR levels. Examination of the impact of herstatin on IGF-I-specific signaling revealed strong inhibition of tyrosine phosphorylation of IRS-1, while IRS-2 activation was enhanced. Although IGF-IR tyrosine phosphorylation was strongly reduced, herstatin expression did not inhibit, but stimulated, IGF-I-mediated ERK activation, while IGF-I activation of the PI3K-Akt/PKB pathway was inhibited. Altered IGF-IR signaling culminated in loss of IGF-I-mediated cell growth and survival in herstatin-expressing clonal cell lines. These studies demonstrate that herstatin profoundly modulates IGF-I-stimulated signaling and proliferation in MCF7 breast cancer cells, either through direct interaction with the IGF-IR or indirectly, by modulating crosstalk with the EGFR family.

Receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR) and the insulin-like growth factor-I receptor (IGF-IR) families, play key signaling roles in fundamental cellular processes. The EGFR family, which includes the EGFR (HER-1/ErbB1), human epidermal growth factor receptor-2 (HER-2/*neu*/ErbB2), HER-3/ErbB3, and HER-4/ErbB4, has been shown to mediate key cellular processes such as growth and differentiation (2-4). The IGF-IR family, which includes the IGF-IR, the insulin receptor, and the insulin receptor-related receptor, has also been shown to participate in an overlapping array of biological processes (5-11). While the expression and biological effects of these receptor families are essential for normal growth and development, aberrant expression leads to a variety of human cancers (12-15).

The four members of the EGFR family each contain an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain (16-18). Eleven growth factor ligands, each containing an EGF core domain, bind with high affinity to these receptors, except HER-2, causing the formation of receptor homo- or heterodimers. This

dimerization results in receptor activation and autophosphorylation *in trans* of specific C-terminal tyrosine residues (4,17,19-22). Phosphorylation of these residues enables the recruitment and tyrosine phosphorylation of SH2-domain-containing signaling molecules, leading to the initiation of two major intracellular signaling pathways: the (generally) anti-apoptotic PI3K-Akt/PKB and mitogenic ERK cascades (12,23,24).

The IGF-IR, in contrast to other RTKs, consists of a pre-formed ($\alpha_2\beta_2$), disulphide-linked, heterotetramer (25,26). Ligand binding is thought to lead to a conformational change in the ß subunits and to activation by autophosphorylation of tyrosine residues in the catalytic domain. The subsequent phosphorylation of additional tyrosines, particularly in the juxta-membrane domain of the ß subunit, provides docking sites for PTB and SH2-domain-containing scaffolding/adapter proteins, including the insulin receptor substrates IRS-1 and IRS-2. These adaptor proteins then activate signaling pathways such as the PI3K and ERK cascades that are also activated by the EGFR family (27).

By virtue of their activation of the PI3K and ERK cascades and potentially other signal transduction pathways, both the EGFR and IGF-IR families are major regulators of cell growth and survival, and dysregulation of either receptor family can lead to uncontrolled growth and tumorigenesis. Recent evidence suggests that there is crosstalk between these RTKs, which may allow coordinated control of cellular responses in normal and tumor cells (reviewed in (1)). Bidirectional crosstalk and coordination of signal transduction between the IGF-IR and EGFR families has been documented (reviewed in (1)). Sustained activation of a mitogenic ERK signal by the EGFR is heavily dependent on a functional IGF-IR (28). Recently, the converse has also shown to be true, in which activation of ERK by IGF-IR requires a functional EGFR (5,29,30). Additionally, it has been shown in several cell types that IGF-I stimulation of the IGF-IR leads to activation of the EGFR and, coordinately, the ERK pathway, through proteolytic activation and autocrine release of HB-EGF (30-32). IGF-I-induced coordinate activation of ERK through EGFR and IGF-IR is in contrast to IGF-I-induced activation of Akt, which is unaffected by EGFR-specific inhibitors (30,32). These data suggest that crosstalk between the EGFR and IGF-IR coordinately controls activation of the ERK signaling pathway, but not the PI3K-Akt/PKB pathway. In addition to coordination of signal transduction, Ahmed et al. have recently reported that the EGFR co-immunoprecipitates with the IGF-IR in mammary epithelial cells, and that phosphorylation of the complexed EGFR is enhanced by treatment with IGF-I (29). More studies, however, are needed to fully elucidate the complex interplay of these receptors.

Because of the important role of the EGFR family in malignant growth, there has been extensive effort directed toward the development and characterization of inhibitors that target these receptors. Effective tumor inhibition has been achieved clinically with inhibitors that antagonize the EGFR and HER-2 (33,34). Several findings support the concept that redundant signaling through IGF-IR maintains the activation of critical pathways for survival in the presence of EGFR family inhibitors. *In vitro*, IGF-IR signaling in MCF7/HER-2 and SKBR-3 breast carcinoma cells protects against inhibition by Herceptin, a therapeutic monoclonal antibody to HER-2 (35). The inhibitory effects of AG1478, an EGFR tyrosine kinase inhibitor, can also be overcome in glioblastoma multiforme cells by overexpression and increased signaling through the IGF-IR (36). Most recently, it has been shown in breast and prostate cancer cell lines that acquired resistance to Iressa, an EGFR small molecule inhibitor, occurs through increased activation and signaling of the IGF-IR (37,38).

While the EGFR family has long been an anti-cancer therapeutic target, recent attempts have also been made at targeting the IGF-IR family. Successful inhibition of tumor growth with two IGF-IR small-molecule inhibitors has been documented with solid tumor xenografts and leukemic malignancies (39,40). A number of specific anti-IGF-IR antibodies have been recently developed that have shown efficacy in inhibition of IGF-stimulated proliferation and tumorigenesis (41-43). Additionally, *in vitro* combinatorial therapy, using Herceptin to block HER-2, and a dominant-negative form of the IGF-IR in breast carcinoma cells, revealed synergy between the two treatments and led to increased growth inhibition (44). Recently, a bivalent monoclonal antibody to the EGFR and IGF-IR has been described (45,46).

Use of this Di-diabody was shown to result in increased growth inhibition compared to that achieved with either anti-EGFR or anti-IGF-IR parent antibodies alone (45). These findings all point to the utility of multi-functional inhibitors that simultaneously target both the EGFR and IGF-IR families.

The current study investigates the impact of a cellular pan-EGFR family inhibitor, herstatin, on IGF-I signaling. Herstatin, the product of alternative splicing of the HER-2 gene transcript, consists of the N-terminal portion of the HER-2 receptor ectodomain, followed by a novel 79-amino acid C-terminal domain (47). Herstatin is unique in that it binds with nM affinity to all members of the EGFR family (48). Herstatin binding to the ectodomain of the EGFR and HER-2 receptors has been shown to block receptor activation (47,49-51). We have recently demonstrated that herstatin also binds, but with reduced affinity, to the IGF-IR compared to the EGFR ($K_d \approx 150$ nM vs 15 nM) (48), presumably to a site in the ectodomain that has homology with the EGFR (52).

In this study, we determine the effects of herstatin, which blocks multiple combinations of the EGFR family, on IGF-I signaling in MCF7 mammary carcinoma cell lines. We also investigate the expression and activation of IGF-IR-specific signaling proteins and IGF-I-mediated proliferation. The results of these studies demonstrate that herstatin, an alternative HER-2 gene product, provides a novel mechanism of cross-regulation between the EGFR and IGF-IR families.

MATERIALS AND METHODS

Cell culture

MCF7 breast carcinoma cells were obtained from the American Type Culture Collection and maintained at 37°C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and gentamicin (0.25 μg/ml). Media and supplements were purchased from Gibco BRL-Life Technologies (Grand Island, NY). Herstatin-expressing MCF7 clones, previously characterized (50), were maintained under the same conditions as parental MCF7 cells in media supplemented with 0.5 mg/ml G418 sulfate.

Antibodies

All primary antibodies were used at a 1:1000 dilution and incubated with Western blots overnight at 4°C, unless otherwise indicated. Polyclonal antibodies [IGF-IRb and IRS-1 (N-terminus)] and monoclonal antibody PY20 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal ERK 1/2 and polyclonal pERK 1/2, Akt/PKB, IRS-1 antibodies were purchased from Cell Signaling Technologies (Boston, MA). Monoclonal herstatin and polyclonal IRS-2 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal pAkt/PKB was purchased from Biosource International (Hopkinton, MA).

Western immunoblotting and immunoprecipitation

Cells were grown to ~80% confluency, serum-starved overnight in DMEM, and treated with 14 nM EGF or 5 nM IGF-I for the times indicated. For Western blots, cells were washed twice with ice-cold PBS and lysed in SDS sample buffer (53) without reducing agent and boiled for 5 min. After clarification by centrifugation at 13,000 rpm for 5 min., supernatant was collected and protein concentration was determined using a detergent-compatible protein assay kit (Bio-Rad; Hercules, CA). Dithiothreitol (100 mM) and bromophenol blue (0.1% (w/v)) were then added and samples were boiled again for 5 min. Twenty-mg aliquots of protein were analyzed by 10% SDS-PAGE and electrotransferred onto nitrocellulose (Amersham Pharmacia Biotech; Piscataway, NJ). Blots were probed with a phospho-specific antibody, stripped in 5x stripping buffer (53) and reprobed with the respective pan antibody. For immunoprecipitation, cells were washed twice with ice-cold PBS, lysed in NP-40 buffer [1% NP-40, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.2% SDS], containing protease inhibitors (Roche Diagnostics; Indianapolis, IN), 1 mM $NaVO_4$, and 1 mg/ml pepstatin. Lysates were cleared and protein concentration was determined as above. For IGF-IR, 1 mg of whole-cell lysate protein was immunoprecipitated with 10 μg of anti-IGF-IR antibody and incubated overnight at 4°C while rocking. For IRS-1 and IRS-2, 500 μg of whole-cell lysate protein was incubated overnight with 5 or 10 μg antibody, respectively. 100 μl of protein A-agarose bead slurry (Amersham Pharmacia

Biotech) was added for 2 hours rocking at 4°C. Three washes were performed, and the pellet was boiled in 2x SDS sample buffer (53). The beads were spun down and the supernatant loaded onto a 10% (IGF-IR) or 7% (IRS-1/2) SDS-PAGE and immunoblotted as above. Blots were probed with PY20, stripped, and reprobed with their respective antibodies. Binding of primary antibodies was detected by enhanced chemiluminescence (Amersham), and film exposures were quantified using a scanning densitometer (Bio-Rad).

Growth assays

Cells ($4x10^4$) were plated in quadruplicate in 24-well plates, incubated in serum-free DMEM for 24 hours, and treated with either 5 nM IGF-I (GroPep; Adelaide, Australia) or an equivalent volume of vehicle (10 mM HCl). At the indicated time-points, cell monolayers were washed with PBS and incubated for 30 minutes at 37°C with 30 µl of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium) inner salt Aqueous One Solution (Promega; Madison, WI) dissolved in 270 ml PBS] per well. Absorbance readings were obtained at 490 nm in a Bio-Tek plate reader.

EGFR inhibitor studies

Control MCF7 cells were serum-starved overnight and treated with the EGFR kinase inhibitor AG1478 (Sigma) or vehicle (DMSO) for 5 min. prior to the addition of 14 nM EGF or 5 nM IGF-I. After 5 min. of growth factor treatment, cell lysates were prepared and analyzed for ERK and Akt/PKB activation as described above.

RESULTS

Effect of herstatin on IGF-IR expression and activation

To evaluate the effects of herstatin expression on activation of the IGF-IR by IGF-I, we examined tyrosine phosphorylation of IGF-IR immunoprecipitated from IGF-I-treated parental and herstatin-expressing cells. In parental MCF7 cells, IGF-I robustly stimulated IGF-IR tyrosine phosphorylation, which represents the initial autophosphorylation stage of IGF-IR activation. In herstatin-expressing cells, however, there was only a small increase in IGF-IR phosphorylation, which corresponds to an approximately 8-fold reduction in activation (Fig. 1). This decreased activation reflects, in part, a decrease in IGF-IR expression consistently seen in herstatin-expressing cells (see Fig. 5), as well as diminished tyrosine phosphorylation (Fig. 1). Reduced IGF-IR expression and activation by IGF-I (and IGF-II) were also observed in a second clonal cell line of herstatin-expressing MCF7 cells (data not shown).

IGF-I activation of IRS-1 and IRS-2

To further investigate the effects of herstatin expression on IGF-I-mediated signaling, we examined the activation of IRS-1 and IRS-2, signaling molecules immediately downstream of the IGF-IR. IGF-I-induced phosphorylation of IRS-1 was severely reduced in MCF7/herstatin cells compared to parental controls (Fig. 2A & B). This decreased tyrosine phosphorylation of IRS-1 was a result of both decreased expression of IRS-1 (~5-fold; see Figure 5), as well as an apparent 6-fold decrease in the efficiency of IRS-1 immunoprecipitation in herstatin-expressing cells. This reduction in the amount of IRS-1 immunprecipitated from herstatin-expressing cells was also seen with a second, N-terminally directed IRS-1 antibody (data not shown). Together, the combined effects of decreased IRS-1 expression and immunoprecipitation efficiency resulted in an ~30-fold difference in the amount of IRS-1 in immunoprecipitates from control and herstatin-expressing cells. This was similar to the difference in tyrosine-phosphorylated IRS-1; therefore, the decrease in IRS-1 protein immunoprecipitated from herstatin-expressing cells was equivalent to the decrease in IRS-1-associated phosphotyrosine. Thus, the relative activation of IRS-1 was similar in control and herstatin-expressing cells.

In contrast, the un-normalized levels of activated (tyrosine-phosphorylated) IRS-2 were actually enhanced by 50% in herstatin-expressing cells, despite the approximately 10-fold reduction in total (and immunoprecipitated) IRS-2 protein seen in herstatin-expressing cells (Fig. 2 C & D & Fig. 5). Thus, herstatin expression resulted in an overall 20-fold increase in IGF-I-stimulated IRS-2 tyrosine phosphorylation when the data are normalized for the decreased IRS-2 expression in herstatin-expressing cells.

IGF-I activation of ERK and PKB

Herstatin has been shown to differentially inhibit EGF-stimulated activation of the Akt/PKB versus the ERK signaling pathway in some cell types (49,54). Similarly, herstatin expression did not inhibit the ERK signaling pathway in IGF-I-treated MCF7 cells. ERK phosphorylation was rapid and transient, with a maximal response at 5 minutes in parental cells. In herstatin-expressing cells, the timing of the maximal response was the same, but the amplitude of total ERK activation, indicated by enhanced phospho-ERK, was enhanced several-fold (Fig. 3). Interestingly, we observed a specific stimulation of ERK2, while there was no change in the activation of ERK1. Furthermore, we consistently observed an increase in the apparent size of ERK1. This may correspond to the appearance of an ERK1 splice variant, or a post-translational modification (55-57). In contrast, IGF-I activation of the PI3K pathway, as assessed by the overall level of Akt/PKB phosphorylation, was reduced by 2-fold in MCF7/herstatin cells (Fig. 4). Thus, herstatin expression in MCF7 breast carcinoma cells does not reduce, but enhances ERK2 signaling, but attenuates the anti-apoptotic Akt/PKB signaling cascade. Similar effects, i.e., enhanced ERK2 activation and decreased Akt/PKB activation, were also seen in a second, independent herstatin-expressing MCF7 clone (data not shown).

Effect of herstatin on the expression of IGF signaling molecules

The studies described above demonstrate the effects of herstatin expression on IGF-I-signaling. Here, we examine the effect of herstatin expression on basal levels of these signaling molecules. The expression of herstatin in MCF7 cells resulted in the down-regulation of several components of the IGF signaling system (Fig. 5). Both IGF-IR and IRS-1 protein levels were decreased 5-fold, while IRS-2 protein levels were down-regulated by 10-fold. There was no apparent difference in the levels of total ERK; however as described above, there was a shift from a preponderance of ERK1 to ERK2, as well as an increase in the apparent size of ERK1, as illustrated in Figure 3. Akt/PKB levels were modestly affected, with an average 2-fold decrease seen in herstatin-expressing cells.

Herstatin reduces IGF-I-stimulated growth and survival in MCF7 cells

Previous studies have shown that stable expression of herstatin in MCF7 breast carcinoma cells blocked heregulin-stimulated proliferation (50). The inhibition of IGF-IR signaling observed in herstatin-expressing cells suggested that herstatin may also interfere with IGF-I-mediated growth and survival. To further investigate the effect of herstatin on IGF-I action, we examined the IGF-I-induced growth of parental MCF7 cells and two clones stably transfected with herstatin, MCF7/Hst#1 and MCF7/Hst#2. Parental MCF7 cells grew in response to IGF-I, whereas cell viability decreased in the absence of growth factor. Both of the MCF7/Hst clones, however, failed to exhibit IGF-I-stimulated growth (Fig. 6).

Herstatin blocks EGF signaling

Previous studies have demonstrated that the EGFR is involved in IGF-I signaling (1,5,29-32). Therefore, the observed effects on IGF-I signaling may have been an indirect effect of herstatin-mediated inhibition of the EGFR. To determine whether EGF-stimulated signaling was attenuated by herstatin, we compared the ability of EGF to activate the ERK and PI3K-Akt/PKB cascades in control and herstatin-expressing MCF7 cells. As shown in Fig. 7, EGF treatment of control cells elicited robust ERK and Akt/PKB phosphorylation, which was severely reduced in cells expressing herstatin. These data demonstrate that herstatin blocks both heregulin and EGF-stimulated signaling in MCF7 cells.

Effect of EGFR inhibition on IGF-IR expression

Herstatin expression had a striking effect on the levels of the IGF-IR. To determine if the observed effects of herstatin on IGF-IR levels were an indirect result of decreased EGFR action, we investigated whether specific inhibition of EGFR mimicked the effects of herstatin. Treatment with the EGFR inhibitor, AG1478, prevented EGF-stimulated activation of ERK (data not shown). However, neither short-term nor long-term treatment with AG1478 resulted in the down-regulation of IGF-IR levels that was seen in herstatin-expressing cells (Fig. 8).

DISCUSSION

An understanding of the effects of herstatin, an autoinhibitor of the EGFR family, on IGF-I signaling is critical to defining the overall mode of action of herstatin and to further clarifying the mechanisms that link the actions of these two important RTK families. Our previous studies have shown that herstatin blocks heregulin signaling and proliferation in MCF7 breast carcinoma cells (50). In the current study, we show that EGF signaling is also blocked in these cells. To further assess the interplay between herstatin and the IGF-IR, initially suggested by binding of herstatin at nM concentrations to the ectodomain of the IGF-IR (47), we examined IGF-I signaling and proliferation in MCF7 breast carcinoma cells in which signaling through the EGFR family is disabled.

We found a striking effect on IGF-I signaling. Foremost, herstatin expression resulted in down-regulation of IGF-IR expression and an 8-fold decrease in IGF-I-induced IGF-IR tyrosine phosphorylation, demonstrating a profound impact on IGF-IR activation (Fig 1). Herstatin expression also resulted in a striking decrease in IRS-1 activation, which is immediately downstream of the IGF-IR in the IGF-I signaling pathway (Fig 2). Most importantly, this altered signaling culminated in a loss of IGF-I-mediated survival of MCF7 breast carcinoma cells that express herstatin (Fig. 6).

In contrast to the blockade of EGF and heregulin-induced ERK activation, IGF-I stimulation of ERK was not inhibited, even though IGF-IR levels were reduced several fold (Fig. 3). Therefore, the extent of IGF-IR activation did not parallel the effects on the downstream ERK signaling cascade. Thus, the low levels of activated IGF-IR appeared to be sufficient to fully activate ERK signaling. Though ERK1 activation was unaffected, we observed a shift in the size of ERK1 in herstatin-expressing cells. We speculate that this size shift may be due to alternative splicing of the ERK1 gene, and may represent the ERK1b splice variant, which is 2.6 kDa larger than ERK1 (55-57). ERK1b has an altered ability to interact with MEK1 and may, therefore, result in a differential signaling profile (56). Further studies are needed to elucidate the cause of the shift in ERK1. Interestingly, in herstatin-expressing cells, we also observed a preferential activation of ERK2 relative to ERK1 (Fig 3). Recent studies have implicated activation of ERK2, but not ERK1, in apoptosis (58-61). Therefore, the preferential activation of ERK2 in herstatin-expressing cells may contribute to the loss of IGF-I-mediated survival demonstrated in Fig. 6.

The effects of herstatin expression on the signaling factors immediately downstream of the IGF-IR, IRS-1 and IRS-2, were complex and distinct. Herstatin reduced both IRS-1 expression and immunoprecipitation efficiency, with a concomitant decrease in IGF-I-stimulated tyrosine phosphorylation (Fig 2 A & B & Figure 5). The mechanisms responsible for the two former effects are unclear. With respect to the differential immunoprecipitation of IRS-1 in control vs herstatin-expressing cells, it is possible that herstatin results in the altered subcellular localization or association pattern of IRS-1, such that the availability of IRS-1 to interact with multiple antibodies in attenuated. One possibility is that nuclear translocation of IRS-1, which has been observed in multiple cell types, including MCF7 cells, is affected by herstatin expression (62). While herstatin expression also resulted in the down-regulation of IRS-2, there was no effect on IRS-2 immmunoprecipitation per se, and IGF-I-stimulated IRS-2 tyrosine phosphorylation was actually enhanced in herstatin-expressing cells, an effect which is very robust when accounting for the decreased IRS-2 levels (Figure 2 A & B & Figure 5). The differential enhancement of IGF-I-stimulated IRS-1 and IRS-2 activation by herstatin may reflect the fact that feedback mechanisms, such as patterns of inhibitory serine phosphorylation, differ between IRS-1 and IRS-2 (63). Interestingly, previous studies have shown that IRS-1, but not IRS-2, is important in IGF-I inhibition of apoptosis, an effect that may underlie the inhibitory effects of herstatin on cell viability seen in the current study (64). Combinatorial effects of herstatin expression that include decreased expression and activation of the IGF-IR and its immediate downstream signaling molecule, IRS-1, reduction in activation of Akt and an increase in activation of ERK2, may all contribute to the retarded growth of herstatin-expressing MCF7 cells (Fig. 5).

There are several potential mechanisms through which herstatin may modulate IGF-IR

signal transduction and, thereby, IGF-I action. First, herstatin may directly bind to intracellular IGF-IR in the secretory pathway; alternatively, secreted herstatin may interact at the cell surface, since we have previously determined that it binds to the ectodomain of the IGF-IR with nanomolar affinity (48). However, since herstatin binds to all EGFR family members, and with higher affinity than to IGF-IR, the impact of herstatin on IGF-I signaling may be indirect and needs to be further investigated in cells that do not express the EGFR family.

A second possibility is that the modulation of IGF-I signaling is a secondary effect due to blockade of EGFR family signaling. Ample evidence exists for an IGF-I-stimulated autocrine loop that results in the release of heparin-binding EGF (HB-EGF) and consequently in the activation of the EGFR (32). To examine whether the effect of herstatin on down regulation of the IGF-IR occurs via the EGFR, we blocked EGFR activation (using the EGFR-specific kinase inhibitor, AG1478) in parental MCF7 cells. While the inhibitor fully blocked EGF-induced ERK activation (data not shown), it failed to mimic the results of herstatin-mediated down-regulation of the IGF-IR (Fig. 8). However, we cannot rule out the possibility that longer-term effects of herstatin expression are involved or that modulation of the other members of the EGFR family indirectly affects IGF-I signaling.

A third possibility is that herstatin may modulate the formation of hetero-oligomers between the IGF-I and EGF receptors. Recent evidence suggests that the EGFR is present in IGF-IR immunoprecipitates, suggesting the interesting possibility that herstatin may disrupt EGFR/IGF-IR hetero-oligomers (29). However, further studies are needed to validate the existence of functional hetero-oligomers between these RTK families. Regardless of whether this mechanism entails a direct or indirect effect of herstatin on the IGF-IR, the results presented here demonstrate a profound modulation of IGF-I signaling by an alternative product of the HER-2 gene.

The roles of both the EGFR and IGF-IR families in neoplastic growth and malignancies have been well documented. Over-expression and autocrine stimulation of both receptor families and their ligands has been implicated in a variety of carcinomas (65-69). Recent evidence in breast and prostate cancer cells has shown that acquired resistance to Iressa, an EGFR tyrosine kinase inhibitor, is mediated by activation and signaling of the IGF-IR (37,38). Furthermore, IGF-IR signaling has been shown to protect HER-2-over-expressing breast carcinoma cells from the inhibitory effects of Herceptin, an anti-HER-2 monoclonal antibody (35). Thus, therapeutic strategies that are directed at both of these signaling systems would be expected to have significant advantages over those that target a single growth factor pathway. Our data suggest that herstatin is an inhibitor that may block proliferative signals from two distinct families of RTKs.

The data obtained in this study were obtained with MCF7 cells and were based on two independent herstatin-expressing clones in comparison to control cells. Although MCF7 cells are a valuable and established model for the study of cellular regulatory mechanisms relevant to breast cancer, it will be desirable to extend these results to other cell types. Constitutive expression of herstatin is, however, toxic to most other cells that we have analyzed; thus, further studies will be facilitated by exploiting conditional, regulated expression models that we are currently developing.

Current receptor-directed therapeutics are typically targeted at a single receptor or receptor family, which may explain, in part, their limited clinical efficacy. Recently, a hetero-bi-functional monoclonal antibody that targets both the EGFR and IGF-IR was found to block both EGF and IGF-I-induced activation of Akt/PKB and ERK, resulting in strong inhibition of xenograft growth (45,46). We suggest that herstatin may have significant promise as a novel anti-cancer agent, since it acts as a multi-functional inhibitor that suppresses signaling from both the EGFR and IGF-IR families of RTKs.

REFERENCES

1. Adams, T. E., McKern, N. M., and Ward, C. W. (2004) *Growth Factors* **22**(2), 89-95
2. Burden, S., and Yarden, Y. (1997) *Neuron* **18**(6), 847-855
3. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) *Embo J* **19**(13), 3159-3167
4. Sweeney, C., and Carraway, K. L., 3rd. (2000) *Oncogene* **19**(49), 5568-5573
5. Kuribayashi, A., Kataoka, K., Kurabayashi, T., and Miura, M. (2004) *Endocrinology* **145**(11), 4976-4984
6. Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., DeAngelis, T., Rubin, R., Efstratiadis, A., and Baserga, R. (1994) *Mol Cell Biol* **14**(6), 3604-3612
7. O'Connor, R., Kauffmann-Zeh, A., Liu, Y., Lehar, S., Evan, G. I., Baserga, R., and Blattler, W. A. (1997) *Mol Cell Biol* **17**(1), 427-435
8. Baserga, R. (1997) *Exp Cell Res* **236**(1), 1-3
9. Baserga, R., Resnicoff, M., D'Ambrosio, C., and Valentinis, B. (1997) *Vitam Horm* **53**, 65-98
10. Morrione, A., Romano, G., Navarro, M., Reiss, K., Valentinis, B., Dews, M., Eves, E., Rosner, M. R., and Baserga, R. (2000) *Cancer Res* **60**(8), 2263-2272
11. Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P. C., Cervera, P., and Le Bouc, Y. (2003) *Nature* **421**(6919), 182-187
12. Blume-Jensen, P., and Hunter, T. (2001) *Nature* **411**(6835), 355-365
13. Holbro, T., Civenni, G., and Hynes, N. E. (2003) *Exp Cell Res* **284**(1), 99-110
14. Pollak, M. N., Schernhammer, E. S., and Hankinson, S. E. (2004) *Nat Rev Cancer* **4**(7), 505-518
15. Rochester, M. A., Riedemann, J., Hellawell, G. O., Brewster, S. F., and Macaulay, V. M. (2005) *Cancer Gene Ther* **12**(1), 90-100
16. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241**(4861), 42-52
17. Schlessinger, J., Ullrich, A., Honegger, A. M., and Moolenaar, W. H. (1988) *Cold Spring Harb Symp Quant Biol* **53 Pt 1**, 515-519
18. Yarden, Y., and Ullrich, A. (1988) *Annu Rev Biochem* **57**, 443-478
19. Dougall, W. C., Qian, X., Peterson, N. C., Miller, M. J., Samanta, A., and Greene, M. I. (1994) *Oncogene* **9**(8), 2109-2123
20. Hynes, N. E. (2000) *Breast Cancer Res* **2**(3), 154-157
21. Riese, D. J., 2nd, and Stern, D. F. (1998) *Bioessays* **20**(1), 41-48
22. Tzahar, E., and Yarden, Y. (1998) *Biochim Biophys Acta* **1377**(1), M25-37
23. Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G. D., and Glass, D. J. (1999) *Science* **286**(5445), 1738-1741
24. Busse, D., Doughty, R. S., Ramsey, T. T., Russell, W. E., Price, J. O., Flanagan, W. M., Shawver, L. K., and Arteaga, C. L. (2000) *J Biol Chem* **275**(10), 6987-6995
25. Ward, C. W., Garrett, T. P., McKern, N. M., Lou, M., Cosgrove, L. J., Sparrow, L. G., Frenkel, M. J., Hoyne, P. A., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Lawrence, L. J., and Tulloch, P. A. (2001) *Mol Pathol* **54**(3), 125-132
26. Massague, J., and Czech, M. P. (1982) *J Biol Chem* **257**(9), 5038-5045
27. O'Connor, R. (2003) *Horm Metab Res* **35**(11-12), 771-777
28. Swantek, J. L., and Baserga, R. (1999) *Endocrinology* **140**(7), 3163-3169
29. Ahmad, T., Farnie, G., Bundred, N. J., and Anderson, N. G. (2004) *J Biol Chem* **279**(3), 1713-1719
30. Gilmore, A. P., Valentijn, A. J., Wang, P., Ranger, A. M., Bundred, N., O'Hare, M. J., Wakeling, A., Korsmeyer, S. J., and Streuli, C. H. (2002) *J Biol Chem* **277**(31), 27643-27650
31. El-Shewy, H. M., Kelly, F. L., Barki-Harrington, L., and Luttrell, L. M. (2004) *Mol Endocrinol* **18**(11), 2727-2739
32. Roudabush, F. L., Pierce, K. L., Maudsley, S., Khan, K. D., and Luttrell, L. M. (2000) *J Biol Chem* **275**(29), 22583-22589

33. Agus, D. B., Gordon, M. S., Taylor, C., Natale, R. B., Karlan, B., Mendelson, D. S., Press, M. F., Allison, D. E., Sliwkowski, M. X., Lieberman, G., Kelsey, S. M., and Fyfe, G. (2005) *J Clin Oncol*
34. Ross, J. S., Schenkein, D. P., Pietrusko, R., Rolfe, M., Linette, G. P., Stec, J., Stagliano, N. E., Ginsburg, G. S., Symmans, W. F., Pusztai, L., and Hortobagyi, G. N. (2004) *Am J Clin Pathol* **122**(4), 598-609
35. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D., and Pollak, M. (2001) *J Natl Cancer Inst* **93**(24), 1852-1857
36. Chakravarti, A., Loeffler, J. S., and Dyson, N. J. (2002) *Cancer Res* **62**(1), 200-207
37. Camp, E. R., Summy, J., Bauer, T. W., Liu, W., Gallick, G. E., and Ellis, L. M. (2005) *Clin Cancer Res* **11**(1), 397-405
38. Jones, H. E., Goddard, L., Gee, J. M., Hiscox, S., Rubini, M., Barrow, D., Knowlden, J. M., Williams, S., Wakeling, A. E., and Nicholson, R. I. (2004) *Endocr Relat Cancer* **11**(4), 793-814
39. Garcia-Echeverria, C., Pearson, M. A., Marti, A., Meyer, T., Mestan, J., Zimmermann, J., Gao, J., Brueggen, J., Capraro, H. G., Cozens, R., Evans, D. B., Fabbro, D., Furet, P., Porta, D. G., Liebetanz, J., Martiny-Baron, G., Ruetz, S., and Hofmann, F. (2004) *Cancer Cell* **5**(3), 231-239
40. Mitsiades, C. S., Mitsiades, N. S., McMullan, C. J., Poulaki, V., Shringarpure, R., Akiyama, M., Hideshima, T., Chauhan, D., Joseph, M., Libermann, T. A., Garcia-Echeverria, C., Pearson, M. A., Hofmann, F., Anderson, K. C., and Kung, A. L. (2004) *Cancer Cell* **5**(3), 221-230
41. Burtrum, D., Zhu, Z., Lu, D., Anderson, D. M., Prewett, M., Pereira, D. S., Bassi, R., Abdullah, R., Hooper, A. T., Koo, H., Jimenez, X., Johnson, D., Apblett, R., Kussie, P., Bohlen, P., Witte, L., Hicklin, D. J., and Ludwig, D. L. (2003) *Cancer Res* **63**(24), 8912-8921
42. Maloney, E. K., McLaughlin, J. L., Dagdigian, N. E., Garrett, L. M., Connors, K. M., Zhou, X. M., Blattler, W. A., Chittenden, T., and Singh, R. (2003) *Cancer Res* **63**(16), 5073-5083
43. Sachdev, D., Li, S. L., Hartell, J. S., Fujita-Yamaguchi, Y., Miller, J. S., and Yee, D. (2003) *Cancer Res* **63**(3), 627-635
44. Camirand, A., Lu, Y., and Pollak, M. (2002) *Med Sci Monit* **8**(12), BR521-526
45. Lu, D., Zhang, H., Koo, H., Tonra, J., Balderes, P., Prewett, M., Corcoran, E., Mangalampalli, V., Bassi, R., Anselma, D., Patel, D., Kang, X., Ludwig, D. L., Hicklin, D. J., Bohlen, P., Witte, L., and Zhu, Z. (2005) *J Biol Chem*
46. Lu, D., Zhang, H., Ludwig, D., Persaud, A., Jimenez, X., Burtrum, D., Balderes, P., Liu, M., Bohlen, P., Witte, L., and Zhu, Z. (2004) *J Biol Chem* **279**(4), 2856-2865
47. Doherty, J. K., Bond, C., Jardim, A., Adelman, J. P., and Clinton, G. M. (1999) *Proc Natl Acad Sci U S A* **96**(19), 10869-10874
48. Shamieh, L. S., Evans, A. J., Denton, M. C., and Clinton, G. M. (2004) *FEBS Lett* **568**(1-3), 163-166
49. Justman, Q. A., and Clinton, G. M. (2002) *J Biol Chem* **277**(23), 20618-20624
50. Jhabvala-Romero, F., Evans, A., Guo, S., Denton, M., and Clinton, G. M. (2003) *Oncogene* **22**(50), 8178-8186
51. Azios, N. G., Romero, F. J., Denton, M. C., Doherty, J. K., and Clinton, G. M. (2001) *Oncogene* **20**(37), 5199-5209
52. Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Zhu, H. J., Walker, F., Frenkel, M. J., Hoyne, P. A., Jorissen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (2002) *Cell* **110**(6), 763-773
53. Sambrook, J., Maniatis, T., and Fritsch, E. F. (1989) *Molecular cloning: a laboratory manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. Staverosky, J. A., Muldoon, L. L., Guo, S., Evans, A. J., Neuwelt, E. A., and Clinton, G. M. (2005) *Clin Cancer Res* **11**(1), 335-340
55. Aebersold, D. M., Shaul, Y. D., Yung, Y., Yarom, N., Yao, Z., Hanoch, T., and Seger, R. (2004) *Mol Cell Biol* **24**(22), 10000-10015

56. Yung, Y., Yao, Z., Aebersold, D. M., Hanoch, T., and Seger, R. (2001) *J Biol Chem* **276**(38), 35280-35289
57. Yung, Y., Yao, Z., Hanoch, T., and Seger, R. (2000) *J Biol Chem* **275**(21), 15799-15808
58. Castro-Obregon, S., Rao, R. V., del Rio, G., Chen, S. F., Poksay, K. S., Rabizadeh, S., Vesce, S., Zhang, X. K., Swanson, R. A., and Bredesen, D. E. (2004) *J Biol Chem* **279**(17), 17543-17553
59. Chen, J. R., Plotkin, L. I., Aguirre, J. I., Han, L., Jilka, R. L., Kousteni, S., Bellido, T., and Manolagas, S. C. (2005) *J Biol Chem* **280**(6), 4632-4638
60. Cheung, E. C., and Slack, R. S. (2004) *Sci STKE* **2004**(251), PE45
61. Frese, S., Pirnia, F., Miescher, D., Krajewski, S., Borner, M. M., Reed, J. C., and Schmid, R. A. (2003) *Oncogene* **22**(35), 5427-5435
62. Morelli, C., Garofalo, C., Sisci, D., del Rincon, S., Cascio, S., Tu, X., Vecchione, A., Sauter, E. R., Miller, W. H., Jr., and Surmacz, E. (2004) *Oncogene* **23**(45), 7517-7526
63. Kim, J. A., Yeh, D. C., Ver, M., Li, Y., Carranza, A., Conrads, T. P., Veenstra, T. D., Harrington, M. A., and Quon, M. J. (2005) *J Biol Chem*
64. Tseng, Y. H., Ueki, K., Kriauciunas, K. M., and Kahn, C. R. (2002) *J Biol Chem* **277**(35), 31601-31611
65. Harari, P. M. (2004) *Endocr Relat Cancer* **11**(4), 689-708
66. Khandwala, H. M., McCutcheon, I. E., Flyvbjerg, A., and Friend, K. E. (2000) *Endocr Rev* **21**(3), 215-244
67. Mendelsohn, J. (2001) *Endocr Relat Cancer* **8**(1), 3-9
68. Slamon, D. J., and Clark, G. M. (1988) *Science* **240**(4860), 1795-1798
69. Yarden, Y., and Sliwkowski, M. X. (2001) *Nat Rev Mol Cell Biol* **2**(2), 127-137

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FIGURE LEGENDS

Figure 1. Herstatin modulation of IGF-I activation of the IGF-IR. MCF7 and MCF7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute time course, and harvested in NP-40 lysis buffer. 1 mg of cell lysate was immunoprecipiated with an IGF-IR antibody and protein A-agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IGF-IR expression and tyrosine phosphorylation using anti-IGF-IR and PY20 anti-phosphotyrosine antibodies, respectively. Western blots were scanned and quantified by densitometry. **(A)** Representative Western blot of IGF-IR immunoprecipitated from IGF-I-treated MCF7 and MCF7/Hst cells. **(B)** A graphical representation of two independent experiments of IGF-I-induced activation of the IGF-I receptor.

Figure 2. The effect of herstatin on IGF-I activation of IRS-1 and IRS-2. MCF7 and MCF7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute time course, and harvested in NP-40 lysis buffer. 1 mg of cell lysate was immunoprecipitated with IRS-1 (A & B) or IRS-2 (C & D) antibodies and protein A-agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IRS expression and tyrosine phosphorylation. Western blots were scanned and quantified by densitometry. **(A)** Representative IRS-1 immunoprecipitation and analysis with anti-phosphotyrosine PY20 antibody. Both light and dark exposures of the IRS-1 immunoprecipitation are shown. **(B)** Graphical representation of 3 separate experiments. **(C)** Representative IRS-2

immunoprecipitation and analysis with anti-phosphotyrosine PY20 antibody. **(D)** Graphical representation of 3 separate experiments.

Figure 3. The effect of herstatin on IGF-I activation of ERK. MCF7 and herstatin-expressing MCF7/Hst breast carcinoma cells were serum-starved and treated with 5 nM IGF-I at 37°C over a 60-minute time course. Cell lysates (50 μg) were separated on a 10% SDS-PAGE gel and then analyzed by Western blotting with ERK and phospho-ERK antibodies. **(A)** Representative Western blot showing IGF-I-induced ERK activation in MCF7 and MCF7/Hst cells. **(B)** Graphical representation of 3 separate experiments.

Figure 4. The effect of herstatin on IGF-I activation of Akt/PKB. MCF7 and herstatin-expressing MCF7/Hst breast carcinoma cells were serum-starved and treated with 5nM IGF-I at 37°C over a 60-minute time course. Cell lysates (50 μg) were separated on a 10% SDS-PAGE gel and then analyzed by Western blotting with Akt and phospho-Akt antibodies. Western blots were scanned and quantified by densitometry. **(A)** A representative Western blot showing IGF-I-induced Akt/PKB activation in MCF7 and MCF7/Hst cells. **(B)** Graphical representation of 3 separate experiments.

Figure 5. The effect of herstatin expression on the expression levels of various signaling proteins. Sub-confluent MCF7 and MCF7/Hst cells were extracted and signaling protein levels were assessed by Western blot. Herstatin expression in MCF7 breast carcinoma cells down-regulated IGF-IR, IRS-1, IRS-2, and pKB/Akt expression, but total ERK expression was unaffected.

Figure 6. The effect of herstatin on IGF-I-stimulated cell proliferation. Parental MCF7 breast carcinoma cells and **(A)** low hst-expressing and **(B)** high hst-expressing clones were serum-starved for 24 hours and then treated with 5 nM IGF-I or vehicle. Growth was determined by the MTS assay as described in Materials and Methods and was assessed at the indicated days.

Figure 7. The effect of herstatin on EGF-stimulated signaling in parental and herstatin-expressing MCF7 cells. Parental (MCF7) and herstatin-expressing (MCF7/Hst) breast carcinoma cells were serum-starved and treated with 5 nM EGF at 37°C for the durations indicated (in minutes). Cells were lysed, and lysates were run on a 10% SDS-PAGE gel and ERK and Akt activation were analyzed by Western blotting as described in the legends to Figures 3 and 4. Western blots were scanned and quantified by densitometry. **(A)** Effect of herstatin expression on EGF-induced ERK activation. **(B)** Effect of herstatin expression on EGF-induced Akt/PKB activation.

Figure 8. The effect of AG1478, an EGFR inhibitor, on IGF-IR expression. MCF7 breast carcinoma cells were treated with the EGFR inhibitor AG1478 at 37°C for the times indicated. Cells were lysed, and lysates were run on a 10% SDS-PAGE gel and analyzed by Western blot. AG1478 had no effect on IGF-IR expression levels over a 24-hour period.

Figure 1.

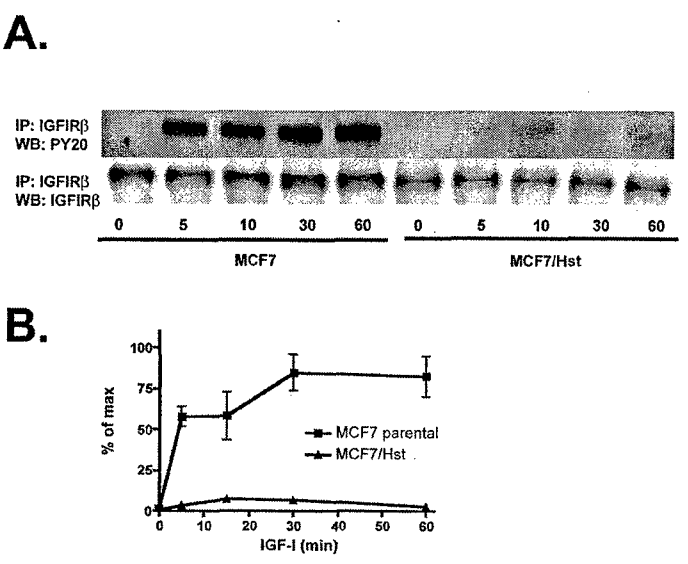

A.
IP: IGFIRβ
WB: PY20
IP: IGFIRβ
WB: IGFIRβ
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
B.
% of max
100
75
50
25
0
0 10 20 30 40 50 60
IGF-I (min)
MCF7 parental
MCF7/Hst

Figure 2.

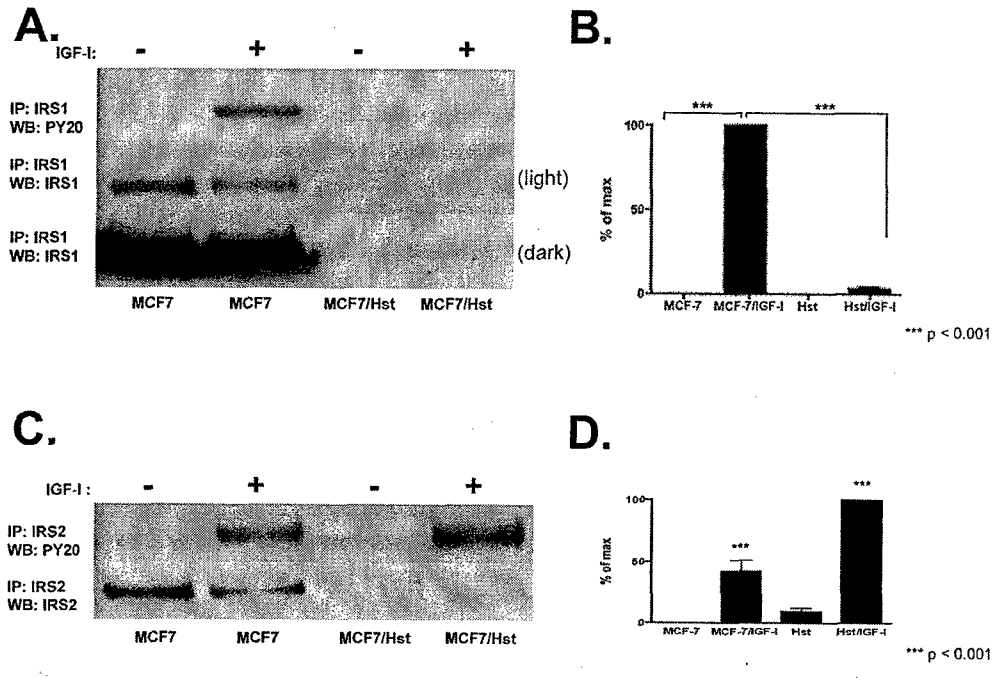

A.
IGF-I: - + - +
IP: IRS1
WB: PY20
IP: IRS1
WB: IRS1
(light)
IP: IRS1
WB: IRS1
(dark)
MCF7 MCF7 MCF7/Hst MCF7/Hst
B.
% of max
100
50
0
MCF-7 MCF-7/IGF-I Hst Hst/IGF-I
*** p < 0.001
C.
IGF-I: - + - +
IP: IRS2
WB: PY20
IP: IRS2
WB: IRS2
MCF7 MCF7 MCF7/Hst MCF7/Hst
D.
% of max
100
50
0
MCF-7 MCF-7/IGF-I Hst Hst/IGF-I
*** p < 0.001

Figure 3.

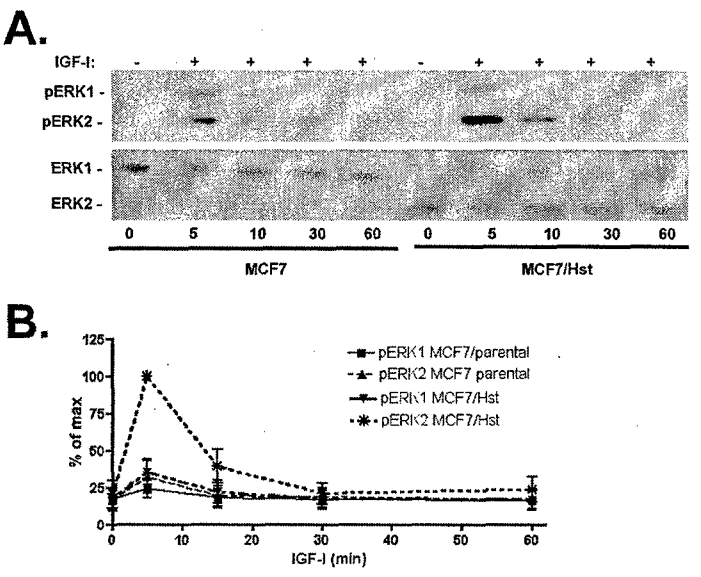

A.
IGF-I: - + + + + - + + + +
pERK1 -
pERK2 -
ERK1 -
ERK2 -
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
B.
pERK1 MCF7/parental
pERK2 MCF7 parental
pERK1 MCF7/Hst
pERK2 MCF7/Hst
% of max
IGF-I (min)

Figure 4.

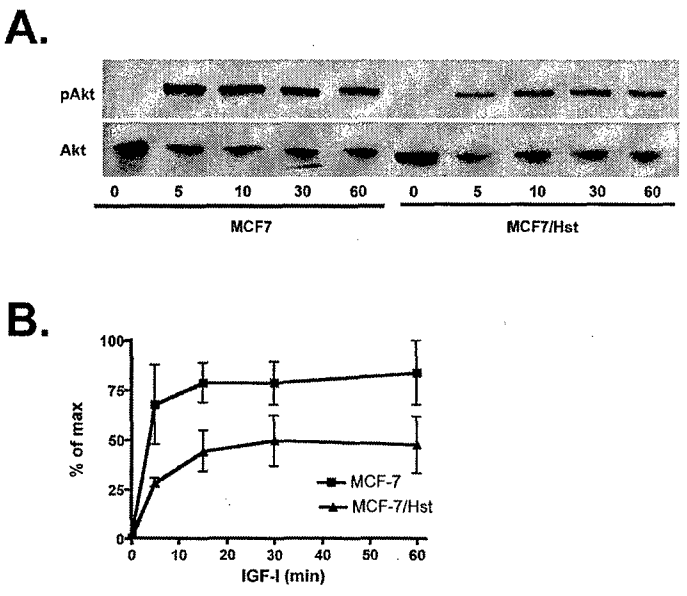
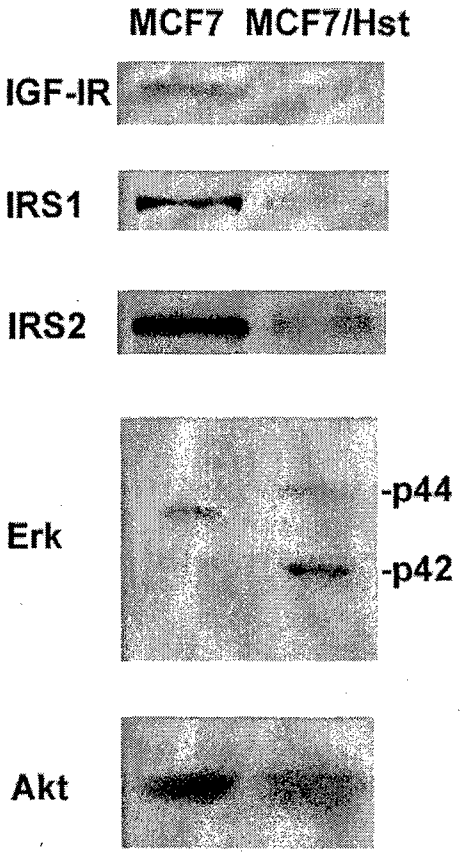

A.
pAkt
Akt
0
5
10
30
60
0
5
10
30
60
MCF7
MCF7/Hst
B.
100
75
50
25
0
% of max
MCF-7
MCF-7/Hst
0
10
20
30
40
50
60
IGF-I (min)

Figure 5.



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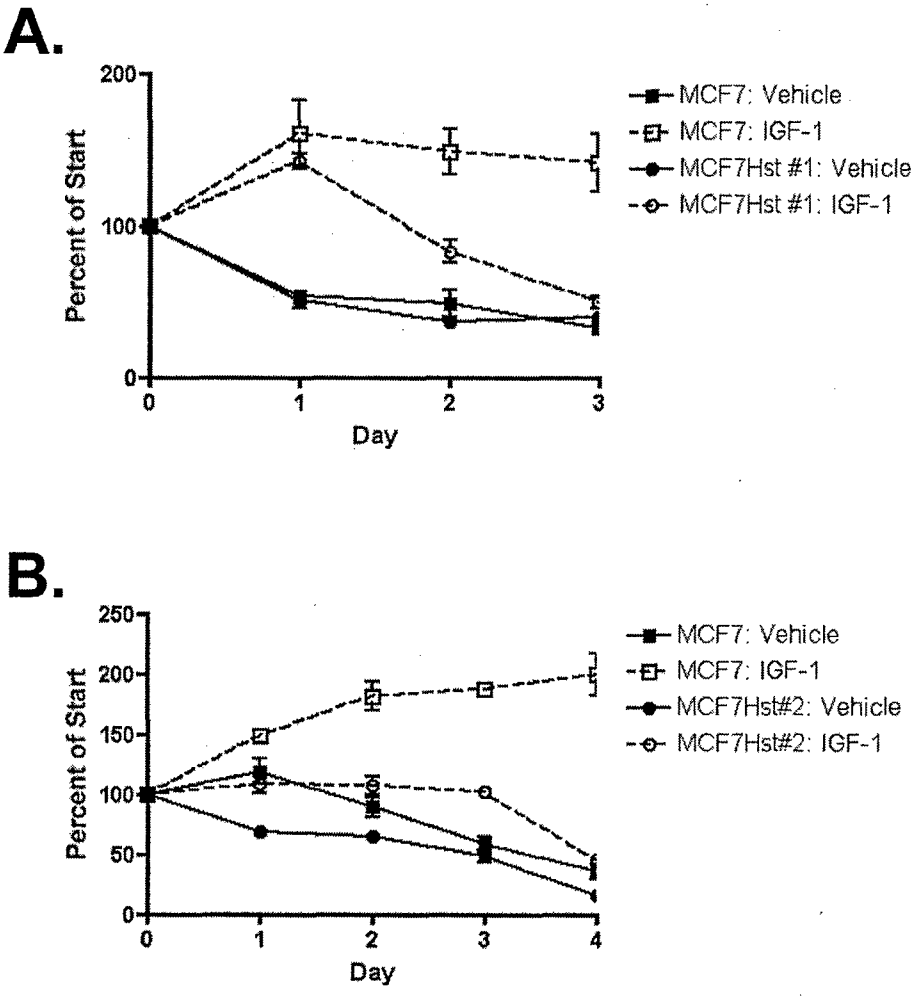

A.
200
100
0
Percent of Start
0
1
2
3
Day
MCF7: Vehicle
MCF7: IGF-1
MCF7Hst #1: Vehicle
MCF7Hst #1: IGF-1
B.
250
200
150
100
50
0
Percent of Start
0
1
2
3
4
Day
MCF7: Vehicle
MCF7: IGF-1
MCF7Hst#2: Vehicle
MCF7Hst#2: IGF-1

Figure 7.

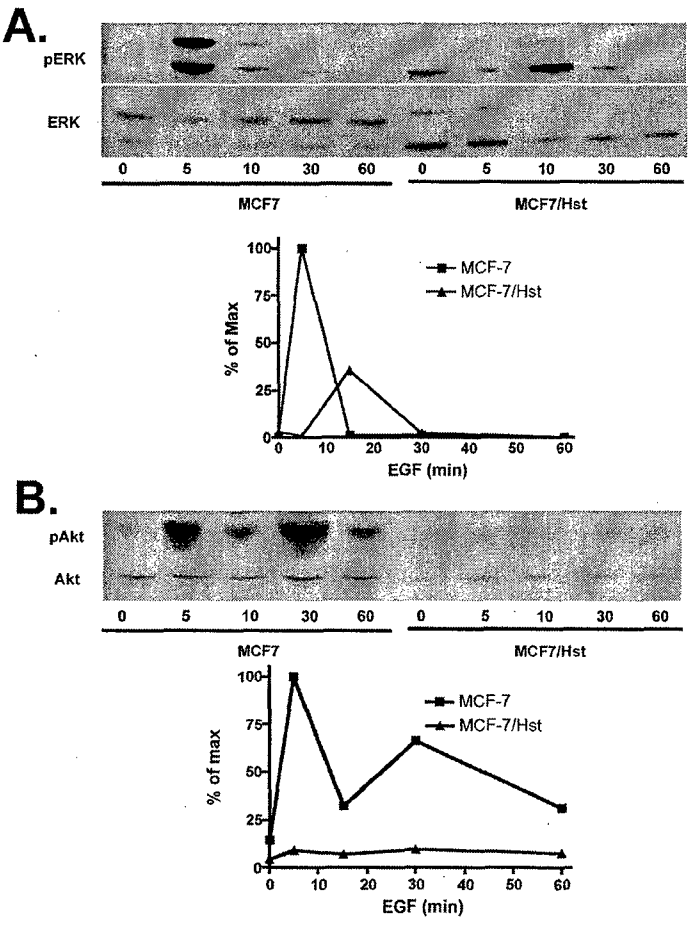
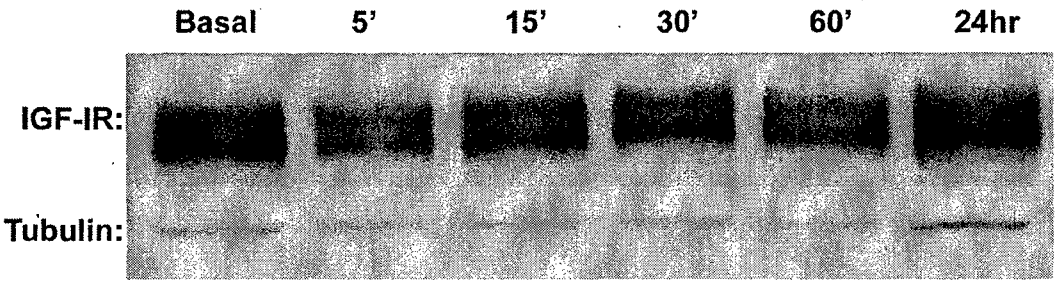

A.
pERK
ERK
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
100
75
50
25
0
% of Max
MCF-7
MCF-7/Hst
0 10 20 30 40 50 60
EGF (min)
B.
pAkt
Akt
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
100
75
50
25
0
% of max
MCF-7
MCF-7/Hst
0 10 20 30 40 50 60
EGF (min)

Figure 8.



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Receptor binding specificities of Herstatin and its intron 8-encoded domain

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Abstract Retention of intron 8 in alternative HER-2 mRNA generates an inhibitory secreted ligand, Herstatin, with a novel receptor-binding domain (RBD) encoded by the intron. This study examines binding interactions with several receptors and investigates sequence variations in intron 8. The RBD, expressed as a peptide, binds at nM concentrations to HER-2, the EGFR, ΔEGFR, HER-4 and to the IGF-1 receptor, but not to HER-3 nor to the FGF-3 receptor, whereas a rare mutation in the RBD (Arg to Ile) eliminates receptor binding. The full-length Herstatin binds with 3–4-fold higher affinity than its RBD, but with ~10-fold lower affinity to the IGF-IR. Sequence conservation in rhesus monkey but not in rat suggests that intron 8 recently diverged as a receptor-binding module critical for the function of Herstatin.

Keywords: ErbB receptor; HER-1, HER-2, HER-3, HER-4; IGF-I receptor; Herstatin

1. Introduction

The ErbB receptor family consists of four receptor tyrosine kinases: EGFR (HER-1, erbB-1), HER-2 (erbB-2), HER-3 (erbB-3) and HER-4 (erbB-4). Aberrant expression of ErbB receptors by mutational activation, receptor overexpression, and tumor production of ligands contributes to the development and maintenance of a variety of human cancers [1,2].

The ErbB receptors are activated by several ligands consisting of an EGF core domain [3]. The exception is the HER-2 receptor, which is recruited as a preferred dimer partner with other ligand binding erbB receptors. While the eleven mammalian EGF-like ligands are all agonists, the ligand Argos, in *Drosophila*, inhibits activation of the EGFR [4,5].

Although the HER-2 receptor does not directly bind EGF-like ligands, a secreted product of an HER-2 alternative transcript, Herstatin, binds with nM affinity to the ectodomain of HER-2. Herstatin consists of a segment of the HER-2 ectodomain followed by 79 novel amino acids, encoded by intron 8, which function as a receptor-binding domain (RBD) [6]. Herstatin blocks homomeric and heteromeric ErbB receptor interactions, inhibits activation of the PI3K/Akt pathway initiated by EGF, TGF-α, and Heregulin, and causes growth arrest suggesting potential as an anti-cancer agent [6–9]. However, no study has yet addressed the receptor specificity of Herstatin. To identify receptor binding targets and to further assess the significance of the novel intron 8-encoded RBD, we investigated binding to several receptors expressed in transfected cells, examined the consequence of a rare mutation in intron 8, and compared the sequence in human, rat and rhesus monkey.

2. Materials and methods

2.1. Cell lines, transfections, and Western blots

The 3T3/HER-2 cells were previously described [10]. The 3T3/IGF-IR cells were from Dr. Charles Roberts, OHSU, Portland, OR. For transient transfections, 2 μg of empty vector or 2 μg EGFR, HER-2, HER-3, HER-4, ΔEGFR, or FGFR-3-myc expression vectors was added with Lipofectamine (Gibco-BRL) to Cos-7 cells in 6 well plates. The HER-2 and EGFR expression plasmids were previously described [7], ΔEGFR was a gift from Dr. Webster Cavenee (Ludwig Institute, UCSD, La Jolla, CA), the FGFR-3-myc construct was from Dr. William Horton (Shriners Research Hospital, Portland, OR), and the HER-4 expression plasmid was a gift of Dr. Nancy Hynes (Friedrich Miescher-Institute for Biomedical Research, Basel, Switzerland). To analyze receptors by Western blot analysis, proteins were resolved by SDS–PAGE and electro-transferred onto nitrocellulose membranes (BioRad, Hercules, CA). Blots were blocked in 5% milk and incubated with primary antibody overnight at 4 °C. The antibodies included anti-HER-2 [11], anti-EGFR, anti-HER-3, and anti-HER-4, which were all rabbit polyclonal antibodies against the receptor C-terminal domains (Santa Cruz Biotechnology). Antibodies against the β-subunit of IGF-IR were from Dr. Charles Roberts. After washing, the blots were incubated with secondary antibody conjugated to HRP for 30 min (BioRad, Hercules, CA). The membranes were developed with Super-Signal West Dura (Pierce, Rockford, IL) and exposed to X-ray film.

2.2. Sequencing of intron 8

Human genomic DNA was obtained from blood samples (supplied by Dr. David Henner, OHSU) from individuals 18 years or more, after giving informed consent, with approval by the Institutional Review Board of OHSU. The samples, assigned random four-digit numbers, could not be traced to patient identity. The polymerase chain reaction (PCR), purification and sequencing were carried out exactly as previously described [6]. Electropherograms were individually reviewed to detect polymorphic alleles. Samples found to contain a polymorphism were sequenced at least twice to confirm the mutation. Rhesus monkey DNA, provided by Dr. Scott Wong (ORPC, Portland, OR), was amplified and sequenced in the same manner. Intron 8 in rat genomic DNA was amplified by PCR using rat specific primers: 5′-CTACCTGTCTACGGAAGTGG-3′ and 5′-TTCCGGGCAGAAATGCCAGG-3′. The cycling parameters were: 94 °C for 30″; 62 °C for 30″; and 72 °C for 60″, for 25 cycles.

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2.3. Expression and purification of intron 8-encoded peptide (Int8) and Herstatin

The intron 8 cDNA was cloned into the pET30 bacterial expression vector (Novagen, Madison, WI), expressed in bacteria (BL-21), and purified by nickel affinity chromatography as described [6]. For purification of insect Herstatin, S2 insect cells, stably transfected with 6× His tagged-Herstatin in the pMT/BiP expression plasmid (Invitrogen, Carlsbad, CA), were induced with 100 μM cupric sulfate for ~16 h. Herstatin was purified to ~90% purity by Ni–NTA (Qiagen, Valencia, CA) affinity chromatography as previously described [8].

2.4. Cell binding studies

About 2×10^6 cells in 6-well plates were incubated with purified Herstatin or int8 peptide for 2 h at 4 °C in serum-free media. Cells were washed with phosphate-buffered saline (PBS) and extracted in 50 mM Tris–HCl, pH 7.0, and 1.0% NP-40. Int8 peptide or Herstatin bound to cells was quantified using a sandwich Herstatin ELISA as per the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). The dissociation constant (K_d) and maximal binding (B_{max}) of Herstatin or the int8 peptide were determined by nonlinear regression analysis of the plot of pmol of bound versus nM of Herstatin or int8 peptide added. Statistical comparisons between different binding curves were performed by extra sums-of-squares *F*-test on nonlinear regression coefficients. All tests were performed ($\alpha = 0.05$) using GraphPad Prism 4 software (GraphPad Software, 1994–2003).

2.5. Pull-downs with int8 peptide immobilized on protein S agarose

About 100 μl of a 50% suspension of S-protein agarose (Novagen) was incubated with or without 100 μg of int8 peptide with an S-protein tag, at room temperature for 1 h, and then washed twice with 500 μl PBS. The agarose samples were then incubated at room temperature for 1 h with 200 μg of transfected Cos-7 cell extract and washed twice with 500 μl of PBS with 1% NP40. The proteins were eluted from the resin at 92 °C for 2 min in 40 μl of SDS-sample buffer and analyzed as a Western blot.

3. Results

3.1. Sequence of human, rhesus monkey, and rat intron 8

Herstatin is generated by retention of HER-2 intron 8, which encodes the unique C-terminal proline-rich domain of 79 amino acids (Fig. 1). Because of its critical function in receptor binding [6], we sequenced genomic HER-2 intron 8 from 214 humans, rhesus monkey, and rat. The HER-2 intron 8 deduced amino acid sequence, originally determined from SKOV3 ovarian cancer cells (AF177761), was found to be the most common in germ line DNA. In addition, we identified a sequence variation in intron 8 (G1112T in AF177761) resulting in an Arg to Ile substitution at residue 31 in Fig. 1. This mutant allele was found in only one of 215 (<0.5%). The deduced amino acid sequence of intron 8 from rhesus monkey was 85% identical to that of humans (Fig. 1) and the nucleotide sequence, up to the stop codon, was 93% identical. However, there was no conservation between rat and human intron 8 (Fig. 1), in contrast to the HER-2 receptor coding sequence, which is highly conserved in rat *neu* [12].

3.2. Receptor binding of the HER-2 intron 8-encoded peptide

To identify other potential receptor targets of Herstatin, we examined binding of the intron 8-encoded RBD, expressed as a bacterial peptide (Int8). Protein S agarose, with or without immobilized int8 peptide, was incubated with extracts from Cos-7 cells transiently transfected with several different receptors. Following washing steps, the protein bound to the agarose was analyzed as a Western blot with receptor-specific antibodies. As previously observed [6,7], EGFR and HER-2 from the transfected cell extracts bound specifically to the agarose with

```
Human: 1                                                  I
Human: 1 GTHSLPPRPAAVPVPLRMQPGPAHPVLSFLRPSWDLVSAF40
Rhesus: 1                          C N     L            P L
Rat:    1 GTQPHSKTSLVHPALAstop

Human: 41 YSLPLAPLSPTSVPISPVSVGRGPDPDAHVAVDLSRYEGstop
Rhesus: 41 C     L        M S       DL          N    C  stop
```

Fig. 1. The deduced amino acid sequence encoded by HER-2 (ErbB-2) intron 8. Alignments are with the most common human intron 8 sequence from 214 individuals with non-conserved residues shown.

int8 peptide (Fig. 2A). In contrast, the int8 peptide with the Arg to Ile mutation at residue 31 (see Fig. 1) did not pull-down the HER-2 receptor (Fig. 2B). Fig. 2A also demonstrates that ΔEGFR, a tumor variant of the EGFR missing its N-terminal subdomains I and II [13], specifically associated with int8 peptide. Another member of the erbB family, HER-4, was also pulled-down by int8. However, there was no detectable association of HER-3 with int8 peptide agarose despite abundant expression in the transfected cells (Fig. 2A). We also investigated the possible interaction with the IGF-1 receptor (IGF-IR), which contains regions of ectodomain sequence homology with the EGFR [14]. Interestingly, we observed specific pull-down of the IGF-IR from transfected cell extracts (Fig. 2A). The FGFR-3, a receptor tyrosine kinase with Ig-like motifs and no structural homology with the ErbB family ectodomains, did not bind to the int8 peptide.

To further examine interaction of the int8 peptide with the extracellular domain of receptors at the cell surface, an Herstatin ELISA was used to quantify bound peptide. In agreement with results obtained by the pull-down assay, the int8 peptide bound in a specific and dose-dependent manner to EGFR, HER-2, HER-4, and ΔEGFR, but not to HER-3, FGFR-3, or mock-transfected cells (Fig. 2C). Binding affinities were further characterized by generating saturation-binding curves. Int8 peptide bound to HER-2 transfected Cos-7 cells ($K_d = 50 \pm 6$ nM) and to EGFR transfected Cos-7 cells ($K_d = 78 \pm 10$ nM) with binding affinities, assessed by comparative nonlinear regression analysis, that were not significantly different ($P = 0.40$) (Fig. 3A). Further, int8 peptide bound to the IGF-IR/3T3 cells ($K_d = 70 \pm 21$ nM) and to HER-2/3T3 cells ($K_d = 66 \pm 16$ nM) with similar affinities ($P = 0.96$) (Fig. 3B). In contrast, the mutant int8 peptide with Arg31Ile did not significantly bind to the HER-2 receptor overexpressing cells at any of the peptide concentrations tested (Fig. 3C) even though the Herstatin ELISA detected the wild-type and mutant peptide equally (Fig. 3D). These results suggested that the int8 peptide bound to EGFR, HER-2, and IGF-1R with overlapping binding affinities and that the Arg-Ile mutation inhibited receptor binding without destroying antibody binding epitopes.

3.3. Receptor binding properties of full-length Herstatin

The full-length Herstatin bound to 3T3/HER-2 cells with a $K_d = 14.7 \pm 1.8$ nM, which is significantly different from the binding affinity of int8 peptide ($P < 0.0001$) by 3–4-fold. A direct comparison of the binding of Herstatin to 3T3/HER-2 and 3T3/IGF-IR cells revealed that the affinity for the IGF-1R ($K_d \sim 151$ nM) was lower ($P < 0.0001$) by about 10-fold (Fig. 4A). The dissociation constant of Herstatin for EGFR was similar to that of HER-2, and was unaffected by ligand occupation indicated by a $K_d = 16.4 \pm 3.6$ nM versus 16.3 ± 3.6 nM (respectively) for Cos-7/EGFR treated or not with 10 nM EGF

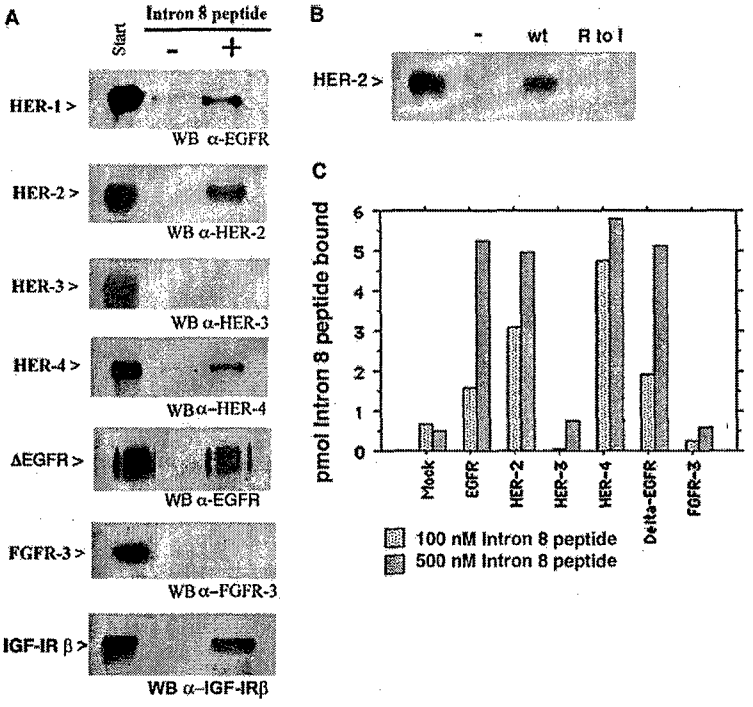

Fig. 2. Binding of intron 8-encoded peptide to different receptors expressed in transfected cells. (A) Extracts from transfected Cos-7 cells were incubated with protein S agarose without or with immobilized wild-type or (B). R31I mutant int8. Associated proteins were analyzed as a Western blot. (C) Transfected Cos-7 cells were incubated with purified int8 for 2 h at 4 °C in serum-free media, cells were washed, extracted, and analyzed by Herstatin ELISA.

(Fig. 4B). Herstatin bound with saturation to endogenous receptors in A431 epidermoid carcinoma cells, which express very high levels of EGFR and low levels of other ErbB receptors (Fig. 4C). At saturation, 6.9 ± 0.4 pmol of Herstatin were bound indicating ~2×10^6 binding sites/cell, which matches the number of EGFR per A431 cell at 2×10^6 [15]. Comparison of nonlinear models indicated that a hyperbolic one affinity-site binding model was the best fit for EGFR-specific binding of Herstatin, in the presence and absence of EGF.

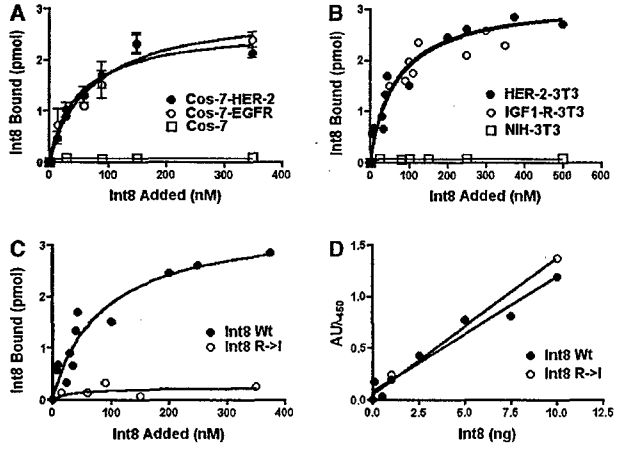

Fig. 3. Saturation binding curves of intron 8 peptide to cells transfected with HER-2, the EGFR, and the IGF-IR. Different amounts of purified int8 were added to the indicated cells and bound peptide was quantified by Herstatin ELISA. Nonlinear regression analysis of binding data was used to determine the dissociation constants (K_d) and maximal amount bound. In (A) parental (Cos7) or transiently transfected Cos-7-HER-2 or Cos7-EGFR cells, or in (B) 3T3 cells or stably transfected HER2-3T3 or IGF-IR-3T3 cells were used. In (C) wild-type or R31I mutant Int8 peptides were incubated with HER2-3T3 cells. In (D) indicated amounts of wild-type or R31I peptides were incubated in an Herstatin ELISA.

4. Discussion

We present evidence that intron 8 of the HER-2 gene, retained in an alternative HER-2 transcript, encodes a receptor binding domain. We also report that a non-lethal, point mutation of unknown physiological significance, resulting in Arg to Ile in the intron 8-encoded domain, eliminates binding to the HER-2 receptor. Unaltered interaction of this mutant RBD with two monoclonal antibodies in an ELISA suggested that global structure was unaffected and that this Arg residue may be directly involved in receptor binding. While the intron 8 encoded domain is critical for receptor binding, it does not appear to affect receptor activity suggesting a requirement for the N-terminal subdomains I and II of Herstatin for receptor inhibition [6] (Shamieh and Clinton, unpublished observations).

While the intron 8-encoded RBD is critical for the receptor binding activity of Herstatin, it is not conserved between humans and rats despite the high degree of sequence identity between the HER-2 receptor and its rat ortholog, *neu*. There are distinct regions in their ectodomains, however, with very little identity [12]. An additional distinction is that the rat *neu* receptor is activated as an oncogene by a single point mutation in the transmembrane domain, while the human ortholog, HER-2, is oncogenic without aberrations in the coding se-

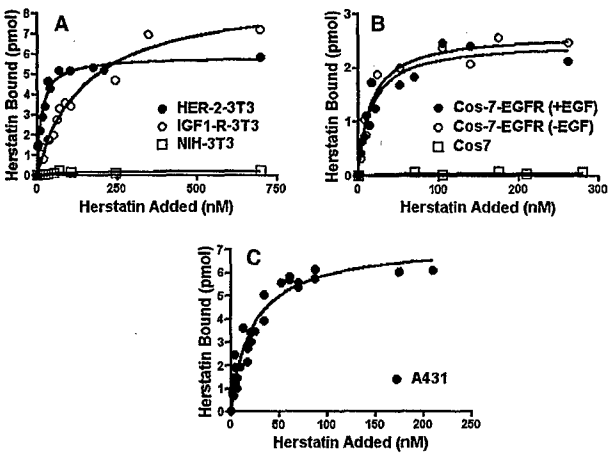

Fig. 4. Saturation binding curves of Herstatin to cells expressing different receptors. Herstatin purified from S2 insect cells was incubated with: (A) 3T3 cells, HER-2-3T3, or IGF-IR-3T3 cells or in (B) with parental or transiently transfected Cos-7-EGFR cells serum starved for 24 h and then treated or not for 2 h on ice with 10 nM EGF, or in (C) A431 epidermoid carcinoma cells.

quence [16]. Furthermore, the activating mutation is not functionally equivalent when introduced into HER-2 [17,18]. These collective observations point to differences in regulation of the human HER-2 receptor versus its rat ortholog, *neu*.

Specific binding of the RBD suggests that the HER-4 receptor will be a target of Herstatin. Since Herstatin binds to and blocks the dimerization of the EGFR and HER-2, we predict that Herstatin will have a similar effect on the structurally similar HER-4. Effects of Herstatin on HER-4 activation and signaling are currently under investigation. Lack of Herstatin binding to the other ErbB family member, the HER-3 receptor, was surprising. HER-3 is unique, however, since it is kinase deficient and requires an active receptor partner to signal. The Herstatin binding site may be disguised when HER-3 is overexpressed without a dimer partner. The binding of Herstatin to the IGF-IR with nM affinity was unforeseen, since ligands do not typically cross-react with receptors from different families. Interestingly, the IGF-IR has regions of ectodomain sequence homology with the EGFR and crosstalk occurs, most notably, with transactivation of the EGFR by IGF-1 [19 and references therein]. Our finding that the binding affinity of Herstatin, but not its RBD, is significantly weaker for IGF-IR than for HER-2 or the EGFR suggests that stabilizing interactions between the N-terminus of Herstatin and the receptor ectodomain are lacking. Since IGF-IR does not have a homologous dimerization loop [14], contacts between the IGF-IR ectodomain and the dimerization arm in subdomain II of Herstatin may be prohibited. The physiological significance of Herstatin binding to the IGF-IR remains to be determined.

In addition to Herstatin, there are several other examples of alternative forms of ErbB receptors that are created by intron read-through [20,21]. Creation of truncated receptors fused to novel C-terminal domains by read-through into introns represents a novel regulatory mechanism important in the diversification of receptor signaling. So far, Herstatin is the only known alternative receptor product that functions as a ligand and is the only mammalian secreted ligand that inhibits the EGF receptor family [18,22,23].

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References

[1] Blume-Jensen, P. and Hunter, T. (2001) Nature 411, 355–365.
[2] Holbro, T., Civenni, G. and Hynes, N.E. (2003) Exp. Cell Res. 284, 99–110.
[3] Groenen, L.C., Nice, E.C. and Burgess, A.W. (1994) Growth Factors 11, 235–257.
[4] Vinos, J. and Freeman, M. (2000) Oncogene 19, 3560–3562.
[5] Jin, M.H., Sawamoto, K., Ito, M. and Okano, H. (2000) Mol. Cell Biol. 20, 2098–2107.
[6] Doherty, J.K., Bond, C., Jardim, A., Adelman, J.P. and Clinton, G.M. (1999) Proc. Natl. Acad. Sci. USA 96, 10869–10874.
[7] Azios, N.G., Romero, F.J., Denton, M.C., Doherty, J.K. and Clinton, G.M. (2001) Oncogene 20, 5199–5209.
[8] Jhabvala-Romero, F., Evans, A., Guo, S., Denton, M. and Clinton, G.M. (2003) Oncogene 22, 8178–8186.
[9] Justman, Q.A. and Clinton, G.M. (2002) J. Biol. Chem. 277, 20618–20624.
[10] Lin, Y.Z. and Clinton, G.M. (1991) Oncogene 6, 639–643.
[11] Christianson, T.A., Doherty, J.K., Lin, Y.J., Ramsey, E.E., Holmes, R., Keenan, E.J. and Clinton, G.M. (1998) Cancer Res. 58, 5123–5129.
[12] Stein, R.A. and Staros, J.V. (2000) J. Mol. Evol. 50, 397–412.
[13] Nishikawa, R., Ji, X.D., Harmon, R.C., Lazar, C.S., Gill, G.N., Cavenee, W.K. and Huang, H.J. (1994) Proc. Natl. Acad. Sci. USA 91, 7727–7731.
[14] Garrett, T.P. et al. (2002) Cell 110, 763–773.
[15] Filmus, J., Pollak, M.N., Cairncross, J.G. and Buick, R.N. (1985) Biochem. Biophys. Res. Commun. 131, 207–215.
[16] Weiner, D.B., Liu, J., Cohen, J.A., Williams, W.V. and Greene, M.I. (1989) Nature 339, 230–231.
[17] Suda, Y. et al. (1990) EMBO J. 9, 181–190.
[18] Hynes, N.E. and Stern, D.F. (1994) Biochim. Biophys. Acta 1198, 165–184.
[19] Ahmad, T., Farnie, G., Bundred, N.J. and Anderson, N.G. (2004) J. Biol. Chem. 279, 1713–1719.
[20] Lee, H., Akita, R.W., Sliwkowski, M.X. and Maihle, N.J. (2001) Cancer Res. 61, 4467–4473.
[21] Reiter, J.L. et al. (2001) Genomics 71, 1–20.
[22] Dougall, W.C., Qian, X., Peterson, N.C., Miller, M.J., Samanta, A. and Greene, M.I. (1994) Oncogene 9, 2109–2123.
[23] Tzahar, E. and Yarden, Y. (1998) Biochim. Biophys. Acta 1377, M25–M37.